# STUDIES ON UROBILIN PHYSIOLOGY AND PATHOLOGY.

## I. The Quantitative Determination of Urobilin.

By ROBERT ELMAN, M.D., and PHILIP D. McMASTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Numerous methods for the quantitation of urobilin have been reported and a great many estimations have been made with their aid in clinical conditions. There has been no semblance of accord among workers as concerns the origin and fate of the pigment; and analysis of the literature[1,2] accounts for this in making clear the conflicting nature of the evidence accumulated. On the whole it is not surprising that interest in the pigment has lagged in recent years despite a general feeling among workers that it has a weighty significance.

The possession of an experimental method[3] adapted to a precise study of bile, an element supposedly of primary importance in the metabolism of urobilin, has led us to take up the study of it. The present paper deals with the quantitative estimation of the pigment in bile, urine, and stool. Subsequent papers will be concerned with its variations under experimental conditions. The term urobilin as used in these studies will be taken to include all of the reduction products of bilirubin capable of causing a green fluorescence in alcoholic zinc acetate solution. With urobilinogen, as such, we have not been concerned, since it is easily oxidized to the more readily recognizable urobilin.

The discovery in 1869 of urobilin was made possible by its spectroscopic properties. Jaffe[4] while examining a specimen of urine noted an absorption band

[1] Meyer-Betz, F., *Ergebn. inn. Med. u. Kinderheilk.*, 1913, xii, 733.

[2] Eppinger, H., Die Hepato-lienalen Erkrankungen, Berlin, 1920, 76.

[3] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

[4] Jaffe, M., *Virchows Arch. path. Anat.*, 1869, xlvii, 405.

lying between Fraunhofer's lines *b* and *F*. He later found it to be more marked in urines from patients suffering from certain febrile diseases. In normal human bile the same phenomenon was observed. From its occurrence in both the urine and bile Jaffe gave the name urobilin to the pigment responsible for the band. He described its presence in normal stools as well,[5] confirming the original observation by Vanlair and Masius.[6]

The interest which the new substance aroused was very great. Investigations of it have been extensive and many workers have described its properties and its relation to the other bile and blood pigments. Without going into detail on the subject, one may summarize what we know of the chemical nature of urobilin in saying that it is a reddish brown pigment derived from bilirubin by reduction in the body, or from urobilinogen by simple oxidation *in vitro*. Urobilinogen, the usual reduction product of bilirubin, is a normal constituent of feces and bile, and, sometimes, in traces, of the normal urine. As the chromogen of urobilin it is spontaneously oxidized to it in the air; and it is identical with the hemopyrrols of the stool.[1,7]

Urobilin is intensely fluorescent in alcoholic zinc acetate solution,[8] characteristically showing a bright green color by reflected light, though in transmitted light it is brown or pink, depending on the concentration. It has characteristic absorption bands, is sensitive to oxidizing agents, and to acids and alkalies, and, in the animal body, probably exists in varying stages of oxidation and polymerization.

### *Previous Methods of Urobilin Determination.*

Previous methods have been reviewed adequately by several authors.[1,9,10] Mention must be made of two, however, which have been largely used in the past. One of these depends on the spectroscopic absorption bands, the other on the development of its fluorescence. By both methods the urobilin-containing solution is diluted until the characteristic manifestation is no longer visible; and the estimation is reported in dilution values. At the outset of our studies both methods were tried and it soon became apparent that the end-point was in both cases highly indefinite. This was especially true of fluorescence, which is scarcely surprising since the threshold of visibility is notoriously so variable. General fatigue,

[5] Jaffe, M., *Centr. med. Wissensch.*, 1871, ix, 465.
[6] Vanlair and Masius, *Centr. med. Wissensch.*, 1871, ix, 369.
[7] Fischer, H., and Röse, H., *Z. physiol. Chem.*, 1912, lxxxii, 391.
[8] Schlesinger, W., *Deutsch. med. Woch.*, 1903, xxix, 561.
[9] Wilbur, R. L., and Addis, T., *Arch. Int. Med.*, 1914, xiii, 235.
[10] Adler, A., *Deutsch. Arch. klin. Med.*, 1922, cxxxviii, 309.

fatigue of the eye, subjective elements, psychic influences all play a part in the results of the determination. We were obliged, therefore, in the beginning, to discard methods based on the threshold of visibility despite the fact that most of the work on record has been performed with their aid. There is one really accurate method of measuring intensity of the fluorescence or of the absorption bands of urobilin by use of the spectrophotometer.[11] But the instrument is elaborate and seemed unadapted to our plans.

Recently, Pincussen[12] has compared fluorescent solutions of urobilin with a standard made by dissolving fluorescein in water. He prepares a series of dilutions of the latter substance, and by comparing them with his unknown specimen arrives at its value in terms of the strength of fluorescein. He mentions the reduction of this value to terms of mg. of urobilin but presents no detailed data thereon.

One can scarcely assume that a solution, because its color is twice as strong as that of another, contains twice the amount of pigment. Even granting a definite relation between intensity of fluorescence and weight of pigment, one cannot say that such curves of fluorescein and urobilin run parallel. Pincussen, furthermore makes no mention of controls on the reaction and on the concentrations of zinc acetate and alcohol, factors which have been shown[13] to influence markedly the intensity of the color.

### *Present Method.*

In the study of urobilin as derived from the body one is confronted with two problems. The first concerns the extraction and separation from interfering pigments, the second the method of quantitation itself. The difficulties of this latter have been indicated in a general way above; they have been overcome, we believe, in the method now to be described.

Our method is, like that of Pincussen,[12] one of comparative fluorescence, but the unknown factor of the relationship of the intensity of fluorescence to content of urobilin has been eliminated. A certain amount of acriflavine is dissolved in water and the zinc acetate filtrate from the urobilin-containing solution is diluted till it matches this color. The standard itself is calibrated against the fluorescence

[11] Charnas, D., *Biochem. Z.*, 1909, xx, 401.

[12] Pincussen, L., *Deutsch. med. Woch.*, 1922, xlviii, 1074.

[13] Marcussen, S., and Hansen, S., *J. Biol. Chem.*, 1918, xxxvi, 381.

of a standard solution of urobilin. The method of obtaining the clear zinc acetate filtrate and acriflavine standard will be described further on.

The fluorescence of urobilin is notably affected by the reaction and the concentrations of the alcohol and zinc acetate employed to develop it. A somewhat detailed investigation in the matter has been made recently by Marcussen and Hansen,[13] who conclude that any concentration of zinc acetate over 2.5 per cent and of alcohol above 50 per cent will bring out the maximum fluorescence, while the point of greatest color is to be found at an acidity equivalent to that of 0.05 N HCl. Our reagents have been so made as to yield approximately these concentrations and this acidity in the final filtrate. Furthermore a standard diluting solution has been used throughout the determinations.

The standard diluting solution consists of 60 per cent alcohol 2,000 cc., zinc acetate 50 gm., and concentrated hydrochloric acid 2 cc., filtered repeatedly till perfectly clear. 15 cc. portions are measured into test-tubes of uniform length and bore, and graduated amounts of the fluorescent solution of urobilin added, say 0.50, 0.55, and 0.60 cc., and the tubes gently shaken to mix the contents. These solutions are compared in color with the standard acriflavine solution placed in test-tubes of the same type and arranged in a row alternately with the unknowns at intervals of 3 cm., in a dark box placed in a room with subdued light. Illumination to show the fluorescence comes from a 200 watt daylight lamp situated 1 meter above the row of tubes, which are examined for their color through a a horizontal opening in one wall of the box opposite the end along which the tubes are arranged in a slotted block fitted to receive them. The axis of vision is thus at right angles to the line of illumination, which makes manifest the fluorescence most advantageously.

The type of box adopted is similar to that described by Adler.[10] The inner walls were painted a dull black and measured 21 by 28 by 16 cm. The eyepiece from an ordinary stereoscope is fitted to the opening, through which the examination of the solutions is made. The slotted block fitted along the other end of the box has six grooves into which 14 mm. tubes are fitted snugly at intervals of 3 cm. A removable cover is provided which contains six round holes bored just over the slots in the block below. Through them the tubes could be inserted or removed at will with the cover intact. They were about 16 cm. long and projected outside 2 to 3 cm. above the box.

The point at which the color match comes closest is noted and the dilution value of the solution under test is calculated. If the unknown, when diluted as indicated above, is still too weakly colored for correspondence with the standard, more of the filtrate is added in 0.10 cc. increases till one of the tubes matches properly. If, on the other hand, the first dilution yields too intense a green, it is advisable to make a preliminary dilution of the zinc acetate filtrate of say 1 to 4, depending upon how deeply fluorescent it is. The dilution factor is, of course, multiplied

into the final value. In this way the error of small additions is avoided; and amounts under 0.30 cc. were, therefore, never used.

### *Calculation of the Dilution Value.*

The dilution value of the fluorescent zinc acetate filtrate must always be multiplied by a factor dependent upon the number of dilutions involved in developing its color. Thus with bile, 8 is the characteristic factor since 20 cc. of the secretion was used, diluted to 80 cc. during clearing and the volume doubled in the final zinc acetate filtrate. With urine it is 2 since each specimen is diluted but twice before its pigment content is measured. When it has first to be cleared as described below, the factor is 5 instead of 2. Similarly with stool specimens, 8 always enters into the final figure. In each case, of course, the value obtained is multiplied according to the amount of the daily specimen.

### *Standard Fluorescent Solution.*

Among a number of fluorescing substances tried, acriflavine—3.6. diamino acridine methochloride,[14] Boots Pure Drug Company—was found best suited to the work. Its solution by direct light has a color not unlike that of urobilin, and its fluorescence on dilution is identical in appearance with that of the pigment. In order to avoid a possible interference by contaminants as well as to increase the delicacy of the reading an end-point of great dilution was adopted. A solution containing 1 mg. in 30,000 cc. of water proved strong enough to give a color that could be used as a standard, giving as it did a well defined green color when viewed in the dark box. Such a solution was found fairly stable under ordinary circumstances if kept away from the light. The parent solution of a 1/1,000 strength was kept in a cool dark place and new dilutions were made up every week, 15 cc. portions of which were placed in the standard tubes at each reading. These solutions were always made up with distilled water which was filtered before using.

### *Separation of Urobilin and Development of Its Fluorescence.*

Before quantitating urobilin according to the method above outlined, it is necessary to recover this pigment from the body fluids containing it and to have it dissolved in alcoholic zinc acetate, for only in such a solution is the fluorescence displayed. The procedures finally adopted to this end are as follows:

*Urine.*—A portion (25 cc.) of the dog's 24 hour specimen was taken, made neutral or barely acid to litmus, and saturated zinc acetate in 95 per cent alcohol was added up to 50 cc., after throwing in about a gm. of the dry powder to insure an excess of the salt. The mixture is shaken and then filtered till clear. A drop or

[14] Benda, L., *Ber. chem. Ges.*, 1912, xlv, 1787.

two of tincture of iodine is added to oxidize all the urobilinogen to urobilin, which is then manifest through the green fluorescence visible by reflected light.

In some instances, as when bilirubinuria is intense, a preliminary clearing may be necessary. The specimen can be cleared the same way as bile by a method to be described further on, but it was found simpler to use the supernatant fluid obtained after a precipitation such as is employed to obtain bilirubin for quantitation.[15] For this purpose calcium bilirubinate is thrown down with calcium chloride in alkaline solution and recovered by centrifugation. The relatively clear supernatant liquid contains practically all of the urobilinogen, as was later found by a comparison with other methods of clearing. After neutralization a 25 cc. portion is treated like an ordinary specimen, as described above. Since this procedure involves a dilution of the original urine sample from 20 to 50 cc. the factor 2½ figures in the final estimation of the dilution value.

*Stool.*—Urobilin is here present as adsorbed matter and the problem is to divide the stool so finely as to enable the extracting solution to withdraw the pigment completely. Adler,[16] investigating this point at some length, found that on simple trituration in a mortar with acid alcohol so large and constant a percentage of the urobilin comes out with the first extraction that for general purposes repeating the process is quite unnecessary. We have preferred to agitate the total stool specimen immediately after its collection with water in an ordinary milk shaker machine. This, within 2 or 3 minutes, breaks up the fecal masses and yields a finely divided emulsion. Each specimen so obtained is made up to 250, 500, 750, or 1,000 cc. with water, depending on its bulk and supposed content of pigment. Aliquot (25 cc.) portions thereof are shaken in a mechanical shaker for an hour with 75 cc. of acid alcohol (1,600 cc. 95 per cent ethyl alcohol, 25 cc. concentrated HCl mixed and made up with distilled water to 2,500 cc.) They are then left overnight at room temperature to allow oxidation of the chromogen to urobilin as suggested by Wilbur and Addis.[9] Since the material is now in alcoholic solution further bacterial action is precluded.

The next day the mixture is shaken and 25 cc. portions are taken, about a gm. of dry zinc acetate is added to each as an excess, and they are made up to 50 cc. with saturated zinc salt in 95 per cent alcohol. Shaking is now done, and the fluid repeatedly filtered till clear. After adding a drop or two of tincture of iodine it is examined for its fluorescence.

*Bile.*—The reported methods of clearing bile by ridding it of bilirubin pigment are numerous, but trial of them has in the past given imperfect results. For the agent if efficient in absorbing the bilirubin as is Fuller's earth[9] or charcoal, holds back urobilin likewise, while if urobilin comes through, as after treatment with calcium hydrate,[17] or talc,[18] the filtrate is deeply yellow with bilirubin.

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[15] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[16] Adler, A., and Schubert, E., *Biochem. Z.*, 1922–23, cxxxiv, 533.

[17] Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.

[18] Auché, A., *Compt. rend. Soc. biol.*, 1908, xlv, 758.

Advantage has been taken, in the method finally adopted, of the extreme solubility of urobilin in ammonia and the adsorbability of flocculent ferric hydroxide for biliverdin as pointed out by Adler.[10] If ferric chloride be added to normal dog bile the color changes from brown to green because of the oxidation of bilirubin to biliverdin. The urobilinogen must simultaneously be changed to urobilin. If now the ferric hydroxide is thrown down as a flocculent precipitate with an excess of ammonia water and filtration immediately carried out a perfectly clear solution comes through, tinged brown or reddish from its content of urobilin, which remains in solution only if the filtrate is ammoniacal. In urobilin-free biles, as will later be shown, the filtrate is colorless.

As routine 20 cc. of 20 per cent ferric chloride is mixed with 20 cc. of fresh dog bile, in a beaker, 40 cc. of 20 per cent ammonia water immediately added, and the mixture stirred and filtered at once into a 50 cc. graduate. When 25 cc. of fluid has been obtained the remainder is discarded. The order in which the reagents are added is important: if the ammonia be added before the ferric chloride the filtrate will not be clear.

To 25 cc. of filtrate concentrated HCl is added drop by drop till the solution is barely acid to litmus, about a gm. of dry zinc acetate is thrown in, and the whole is made up to the 50 cc. mark with saturated zinc salt in 95 per cent alcohol. The filtrate from this mixture will show the green fluorescence of the urobilin contained in the original bile sample.

### *Expression of Findings in Terms of Mg.*

To express the findings in mg. it was necessary to determine the dilution value of a known amount of pure urobilin examined under the ordinary conditions in the standard dilutant. After repeated attempts a specimen of high purity was obtained as follows:

Heavily pigmented dog stools were selected and extracted with ether to remove most of the fatty acids, and lipoids, indole and scatole. The residue was then ground up and whirled in a motor-driven milk shaker with acid alcohol, agitated in a mechanical shaker for an hour, and centrifuged. The clear supernatant brown liquid was poured off and one-third its volume of chloroform added. This gave a clear mixture. Shaking in a separatory funnel with water brought down the lower chloroform layer and this was washed once or twice with water, passed through a filter paper in a funnel containing anhydrous sodium sulfate to remove the water, and evaporated over a warm water bath, in an Erlenmeyer flask. The dark sticky residue was taken up in 20 per cent ammonia water with which it formed a clear solution, and ice was added. Precipitation of the urobilin was then effected by the slow addition of concentrated sulfuric acid while the solution was kept cold by further additions of ice. The formation of ammonium sulfate is known to throw down the pigment completely as the solution becomes acid. The

flocculent precipitate thus formed was filtered off, washed several times with water, and allowed to dry.

This residue was obviously impure, still having a faint fecal odor. It was washed with ether which extracted lipoids as well as a good deal of pigment other than urobilin; and, after drying again, it was extracted with chloroform, filtered, and allowed to evaporate on a large watch crystal over a warm water bath. The dry film of urobilin scraped off had the form of thin glistening, non-crystalline plates. It was perfectly hard and dry, and after grinding in a small agate mortar yielded a homogeneous, impalpable, odorless powder of dark brown color. It was soluble in alcohol and chloroform, insoluble in ether, wood alcohol, and benzene. It could not be dissolved by water but if a few drops of ammonia water were added it went immediately into solution.

The urobilin thus prepared was assumed to be pure, and, however this may have been, it was sufficiently so to serve as a standard in the work for we have been concerned only with relative changes in urobilin values. The absolute determination of this pigment has been, for us, of little significance. The purified substance was intensely fluorescent, a tiny particle in alcoholic zinc acetate giving rise to a most intense green color. In dilution values 1 mg. in 950 cc. of the standard dilutant matched the fluorescence of the acriflavine standard, which contained 1 mg. of this substance in 30,000 cc. of distilled water, the end-point selected for the final comparison throughout all the determinations.

It was easy now simply to divide all dilution values by 950 and obtain an equivalent in approximate if not actual mg. of urobilin. Thus the possibility of error due to changes in the pH, alcoholic or zinc acetate concentrations was eliminated. The final estimation of color in every case, including that of the pure urobilin pigment itself, was performed in the same solution and under the same conditions. The expression of the values in terms of mg., therefore, involves no correction due to these factors.

Duplicate and repeated readings of the same urobilin specimen were carried out and agreement within 10 per cent was found to be the rule. When a zinc acetate filtrate matched the standard directly, that is to say, without any dilution there can have been present in it only 1 mg. of urobilin for every 950 cc. or, if one take a concrete case, in a bile sample of 100 cc. less than 1 mg. Specimens containing amounts under this could not be quantitated, but traces were roughly estimated by the use of a concentrated beam of light. The routine use of the strong light beam gave proof of the presence or absence of the pigment. Urobilin-free solutions were those which showed no color even with intense illumination.

## DISCUSSION.

The difficulties of urobilin quantitation are considerable, as has already been sufficiently pointed out. There has been no simple, clinically useful method free from objections. It is very largely this absence of a satisfactory means for measurement which has retarded precise investigations of the pigment. In the method outlined above a degree of accuracy has been attained, it is believed, which enables one to estimate the amount of pigment satisfactorily; though exactitude cannot be expected until more is known of its chemical nature. Fortunately exactitude is not necessary for studying the physiology and pathology of urobilin. It is sufficient to be able to determine whether the substance is present, and to follow with a fair degree of accuracy its relative variations. These requirements have been met in the methods devised by us. Filtrates matching our standard solution contain only 0.009 mg. of urobilin per cc. The substance is fluorescent under a concentrated beam of light in far greater dilution than this, so great indeed that when the phenomenon cannot be observed one may safely conclude that to all intents and purposes urobilin is absent.

Some of the urobilin must be lost in the extraction and clearing methods. In the case of the stool not more than 10 to 20 per cent is left behind after a mere trituration;[10] and far less can have escaped our method of treatment. Little is lost in the urine because it is usually examined directly. But with bile the loss may be considerable. A comparison of various methods of clearing leads us to believe that over 75 per cent of the urobilin is recovered from specimens cleared with ferric hydroxide. As already mentioned, duplicate determinations were made on a large series of specimens and agreement within 10 per cent was always the result. 1 or 2 days delay in reading, made no difference when the specimen had been kept in a stoppered vessel in a dark cool place.

The value of fluorescence as an ultimate measure of urobilin depends on the manner in which it is utilized. For the difficulties encountered when this is not properly done one may consult the recent discussion between Adler[19] and Pincussen.[20] In the method here presented

[19] Adler, A., *Deutsch. med. Woch.*, 1922, xlviii, 1442.

[20] Pincussen, L., *Deutsch. med. Woch.*, 1922, xlviii, 1443.

only one assumption has been made; that two urobilin solutions examined under the conditions we have described and showing the same degree of fluorescence, on comparison with an acriflavine standard, contain the same amount of pigment.

In the ultimate quantitation of the material it would have been best, of course, to use weighed amounts of urobilin itself as the standard. This substance is, however, too unstable for use over long periods of time, and moreover its preparation is difficult and tedious. Acriflavine is a convenient and satisfactory substitute. The fluorescence is stable and practically identical in hue with that of urobilin. No difficulty at all was experienced in making a color match.

The method has proved easy to carry out and ideally suited for routine laboratory estimations on material from the dog. Whether it will prove equally suited to clinical purposes remains to be determined.

## SUMMARY.

Methods are presented for the development of clear fluorescent solutions from urobilin-containing urine, feces, and bile obtained from the dog. Conversion of the urobilinogen to urobilin is accomplished during the procedures. Measurement of the urobilin content is effected by comparing its fluorescence at great dilution with a standard containing acriflavine, calibrated in turn against pure urobilin.

Means have been found to avoid the influence of the factors responsible in part for the inaccuracy of urobilin determinations. The method will quantitate exceedingly small amounts of urobilin and estimations on the same material agree within 10 per cent.

Though designed for experimental procedures on the dog, the methods would seem adapted for clinical uses as well.